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CONTACTS CLOSER THAN 4Å – SEQUENCE OF BPTI
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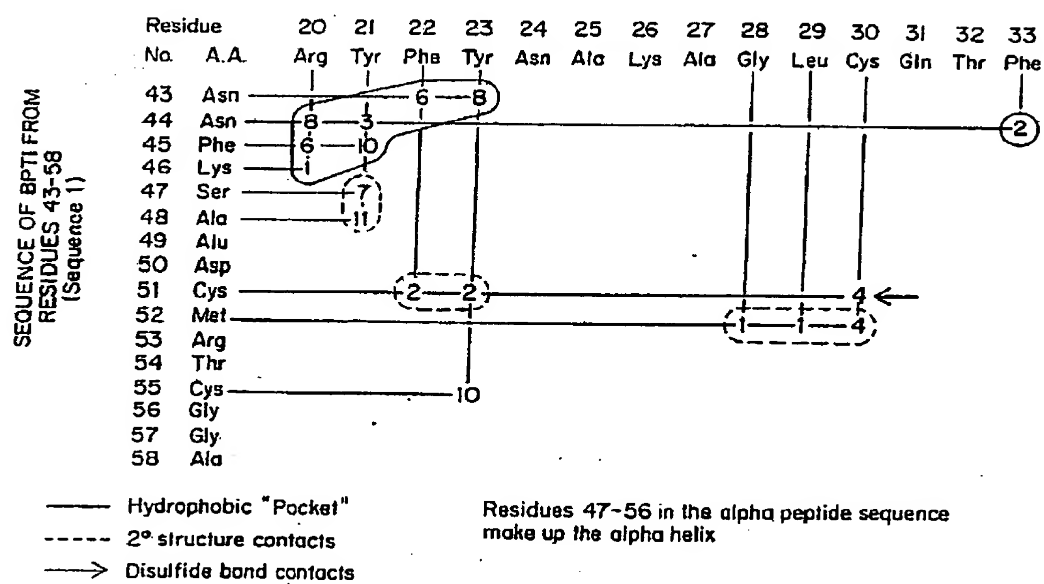
(Sequence 2)



(57) Abstract

Peptide complexes comprising at least two short peptide units which correspond in amin acid sequence to the amino acid sequences of segments of an intact protein of interest which form defined secondary structure in the intact protein and methods of their preparation. The peptide units in the peptide complexes are generally approximately 5 to 30 amino acid residues in length and joined by covalent bonding, for example disulfide bonds formed between a cysteine residue in each short peptide unit.

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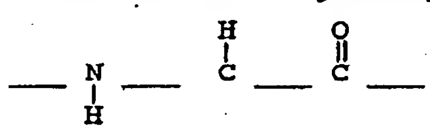
PEPTIDE COMPLEXES HAVING ENHANCED STABILITY

Description

Background

Proteins have a key role in essentially every biological process. For example, they serve as enzymes, function in transport and storage of many small molecules and ions, control expression of genetic information, are critical in immune response to and protection against foreign substances and are themselves antigenic (i.e., their introduction into a host which recognizes them as foreign or nonself elicits an immune response).

The basic structural components of proteins are amino acids, each of which consists of a carbon atom (an alpha carbon), to which is bonded an amino group, a carboxyl group, a hydrogen atom and a distinctive side chain or R group. Amino acids are linked by peptide or amide bonds, in which the alpha-carboxyl group of one amino acid is joined to the alpha amino group of a second amino acid, to form polypeptide chains. Each amino acid unit within a polypeptide chain is referred to as an amino acid residue. The main chain or backbone of a polypeptide chain is the regularly repeating unit



of the chain; in addition, there is a variable part of the chain, which is made up of the distinctive side chain or R group of each amino acid in the polypeptide. The polypeptide chain generally has considerable flexibility,

which results from free rotation of atoms around the bonds between them.

As a result, a protein molecule, which is made up of one or more polypeptide chains, can, in theory, take on any one of many different three-dimensional shapes or conformations. However, most polypeptide chains fold into only one of the possible conformations and the conformation a protein assumes under biological conditions is determined by the amino acid sequence of the polypeptide chain(s) making up the protein. For example, interactions between the amino acid side chains and between amino acids and water contribute to the force which gives one conformation of a protein particular stability. Important factors contributing to the conformation taken by a given protein are the occurrence and distribution of polar and nonpolar side chains and the formation of disulfide bonds (or S-S bridges) between cysteine-SH groups close to one another in a folded polypeptide chain. Generally, the result is that a protein molecule folds spontaneously into a characteristic and unique conformation (e.g., compact and globular or long and fibrous).

Although the total conformation of each protein molecule is unique, there are several patterns of folding which have been found to occur repeatedly. Two patterns occur particularly often because they are the result of hydrogen bonding between peptide bonds in a protein, rather than of side chain interactions.

The first of these two folding patterns, called an alpha-helix, results when a polypeptide chain turns regularly around itself. This results in a rigid cylinder or rod, in which the coiled peptide chain is the

inner part of the rodlike structure and the amino acid side chains or R groups extend outward. Hydrogen bonds between NH groups and CO groups of the main peptide chain stabilize the alpha-helix.

The second of the folding pattern, called a beta pleated sheet, is very different from the alpha-helix. For example, the former is a sheet in which the polypeptide chain is almost fully extended and the latter is tightly coiled. Stabilization of beta-sheets results from hydrogen bonds between NH groups and CO groups in different polypeptide chains in the protein molecule. Adjacent strands in a beta-sheet can be parallel (i.e., they run in the same direction) or anti-parallel (i.e., they run in opposite directions).

Proteins have been shown to exhibit different "levels" of organization structurally. A protein's amino acid sequence is the first level; this is often referred to as the protein's primary structure. The second level of protein folding or structure is formed by hydrogen-bond interactions of amino acids which are located close to one another. The alpha-helix and the beta-sheet are examples of secondary structure; many other types of secondary structure have been defined, on the basis of the dihedral angles formed between adjacent peptide bonds. The third level of protein folding (tertiary structure) is formed by interactions between amino acid residues which are far apart in the amino acid (linear) sequence. Finally, quaternary structure refers to the manner in which the multiple polypeptide chains which make up a protein are packed together.

The critical determinant of a protein's function is its conformation, or the three-dimensional arrangement of

its components. It appears that proteins fold, and achieve their characteristic conformations, by the association or interaction of short regions or stretches in them which transiently assume an alpha-helical or beta-sheet form.

In general, short peptides which have the amino acid sequence corresponding to the amino acid sequence in a region of a native or intact protein do not, under physiological conditions, assume the same conformation as does the equivalent region in the native protein. As a result, peptides or protein "fragments" usually do not have the same functional characteristics (e.g., as enzymes, antibodies, antigens, etc.) as those evidenced by the intact fragment (the equivalent region in the complete or native protein).

Considerable effort has been made to develop short chains of amino acids, or polypeptides, which fold into the native structure of the whole protein, in the hope of producing polypeptides with stable structure and the functional characteristics of the intact protein. This has received particular attention in the area of synthetic vaccine development, but with limited success. For example, Lerner and co-workers and Doolittle and co-workers describe synthetic vaccines which are synthetic peptides/short protein chains designed to mimic a very small region of the outer coat of a virus. They have attempted to select short amino acid sequences which occur in regions of the virus (i.e., the surface of the folded protein antigen) which elicit an immune response when introduced into a host and to determine whether antibodies to a peptide will react with the entire virus.

For example, in U.S. 4,544,500, Bittle and Lerner describe development of synthetic peptides corresponding in amino acid sequence to specific regions of the foot and mouth disease virus (FMDV) VP_1 protein. In particular, single short peptides (i.e., approximately 20 amino acids in length) corresponding in sequence to positions 130-160 and positions 141-160 of the FMDV VP_1 protein are described as "extremely active antigenically."

Richman and Reese report that a 28-residue peptide has been synthesized that will raise antibodies against the 75-KDa surface protein of the malarial parasite Plasmodium falciparum. Richman, S.J. and R.T. Reese, Proc. Nat'l Acad. Sci. USA, 85, 1662-1666 (1988).

On the basis of this work, considerable effort has been made to produce antibodies using individual or single peptide fragments of proteins which bind tightly to the corresponding intact protein.

Such efforts have, however, been relatively unsuccessful, probably because these short peptides fail to adopt a stable structure similar to that of the native protein under physiological conditions. A means by which short peptides could be constructed such that they "mimic" the equivalent region of the native or intact protein, not only in terms of amino acid sequence (primary structure), but also in terms of secondary structure (e.g., alpha-helix or beta-sheet formation), folding, and conformation, would be very useful in synthetic vaccine development and many other areas in which protein structure is a key consideration.

Disclosure of the Invention

Peptide complexes which include at least two short peptide units corresponding in amino acid sequence to selected regions of a protein of interest and joined in such a way that the short peptide units interact with one another in much the same manner that the regions of the native or intact protein interact are the subject of the present invention, as are methods of their preparation and use.

Short peptide units, each generally having approximately five to thirty amino acid residues and corresponding in amino acid sequence to distinct regions of a protein of interest, have been joined by covalent bonding of the short peptide units to produce peptide complexes which are more stable, under physiologic conditions, than presently-available peptides, which are individual or single peptides. In one embodiment of the present invention, two short peptide units, each including approximately five to 30 amino acid residues and corresponding in sequence to sequences in an intact protein which form defined secondary structure and, optionally, to sequences which correspond to sequences in other regions of the intact protein, are joined by covalent bonding. The resulting peptide complex forms a structure more stable than either of the two peptide components alone.

In a particular embodiment of the peptide of the present invention, two short peptide units, each including a cysteine residue and corresponding to a distinct region of bovine pancreatic trypsin inhibitor (BPTI), have been joined by means of a disulfide bond between the cysteine residue present in each. One of the peptide units has 16 amino acid residues, including a cysteine

residue; all or a portion of the unit corresponds in sequence to a distinct region of bovine pancreatic trypsin inhibitor (BPTI) which folds into an alpha-helix in the intact protein. The other short peptide unit has 14 amino acid residues, also including a cysteine residue. It corresponds in sequence to a distinct region of bovine pancreatic trypsin inhibitor which folds into a beta-sheet in the intact protein. The amino acid sequence of the synthetic peptide pair is homologous to a domain of the precursor of the amyloid Beta-protein characteristic of Alzheimer's disease which contains a protease-inhibitor sequence. The resulting peptide complex has been shown, using the temperature dependence of circular dichroism, nuclear magnetic resonance and ultraviolet absorbance spectra as criteria for structure formation, to be more stable than individual short peptides. It has been shown not only that, as expected, the individual short peptides show little, if any, structure formation in aqueous solution, but also that the peptide complex is more stable in aqueous solution than individual short peptides.

Peptide complexes of the present invention can be used in the design and production of synthetic vaccines which are peptides whose sequence mimics portions of that of an intact or native protein antigen. In this context, the peptide complexes are particularly valuable because they form conformations or structures which are stable under physiological conditions. Such synthetic vaccines can be administered to an individual (e.g., human, other mammal or nonmammalian animal) in whom an immune response is desired.

In a similar fashion, peptide complexes of the present invention can be used as artificial proteins in a therapeutic, prophylactic or diagnostic context. For example, they can be used as drugs or in place of naturally-occurring proteins, such as enzymes and hormones. For example, a peptide complex of the present invention can be constructed to incorporate the functional component(s) or active site(s) of a naturally-occurring enzyme, alone or in combination with other selected regions of the enzyme.

In addition, peptide complexes of the present invention can be used for diagnostics. For example, a selected peptide complex of the present invention can be used to produce peptide antigens which are more suitable for use in diagnostic tests than are presently-available peptide antigens because of the similarity of the behavior of the peptide complex of the present invention to that of the intact/native protein against which an immune response is mounted in the body. Such peptide complexes can also be used to produce antibodies capable of reacting with the complex and with the corresponding section of the intact/native protein. Such antibodies also have diagnostic applications.

Synthetic peptides of the present invention can, because of their homology to an Alzheimer's amyloid protein, be used to produce a diagnostic for detecting the presence of the amyloid protein in tissue and, thus, of the disease. In this case, each of the two short peptide units in the peptide complex corresponds to a region of BPTI, as described previously. Antibodies against the peptide complex can be produced, using known

techniques. Peptide complexes of the present invention are also useful for mapping and/or screening discontinuous epitopes. Peptides representing discontinuous epitopes are difficult to synthesize. The present invention is useful in the design and construction of peptides to discontinuous epitopes and in construction of synthetic ligands. Synthetic ligands made according to the method of the present invention can be used, for example, to interfere with the ability of an infectious agent (e.g., human immunodeficiency virus, HIV) to bind to a cell receptor (e.g., on T4 lymphocytes) and, thus, prevent infection. In this case, it is possible to determine the characteristics of the receptor or site on a cell with which the infectious agent interacts and, using the method of the present invention, design and produce synthetic analogues which interact with either the cell receptor or the site on the infectious agent which normally interacts with the cell in the infectious process and interfere with the infectious agent's ability to infect cells. It is also possible, using the method of the present invention, to design and produce peptide complexes which include the catalytic subdomains or regions of a protein. For example, it is possible to determine the catalytic subdomain, or enzyme subdomain, of an enzyme of interest and produce a peptide complex of two or more short peptide units which will exhibit the enzymatic activity of the intact or native protein. In a similar manner, it is possible, using the method of the present invention, to produce a synthetic analog of an immunogenic protein which is less immunogenic than the intact or native protein. This can be done by determining the necessary characteristics and sequence of

the protein binding sites and producing a peptide complex which has similar characteristics but lacks the remainder of the protein, which is responsible for the immunogenicity.

Brief Description of the Drawings

Figure 1 represents an approach to the design of peptide complexes of the present invention.

Figure 2 is the ^{1}H NMR spectrum of the alpha/beta peptide complex of bovine pancrease trypsin inhibitor (BPTI).

Figure 3 is the ^{1}H NMR spectrum of the aromatic region of the alpha/beta peptide complex of BPTI. Resonances due to protons bonded to aromatic carbons in the side chains of tyrosine or phenylalanine are found in this region.

Figure 4 is a graphic representation of the chemical shifts of aromatic peak A in the ^{1}H NMR spectrum of the alpha/beta peptide complex (See Figure 3) at various temperatures.

Figure 5 is a graphic representation of the chemical shifts of all assigned amide and alpha protons of the alpha/beta complex ($P\alpha P\beta$) versus those observed for the corresponding residues in BPTI. The range of chemical shift values for amide and alpha protons in model "random coil" peptides shown with squares.

Figure 6 presents circular dichroism spectra of the alpha and the beta peptides and the alpha/beta peptide complex.

Figure 7 presents the circular dichroism ellipticity at varying temperatures and a wavelength of 222 nM of the

alpha and the beta peptides and the alpha/beta peptide complex.

Figure 8 is a graphic represention of the temperature dependence of the CD signal at 218 nm for the alpha/beta complex in the presence or absence of guanidine hydrochloride (GuHCl). The inset shows the first derivative of the temperature dependence in the absence of GuHCl.

Figure 9 presents ultraviolet absorbance due to tyrosine at varying temperatures and at 285.5 nM.

Figure 10 compares the amino acid sequence of the amyloid precursor protein (APP) domain with the amino acid sequence of the BPTI alpha/beta complex of the present invention and also presents the amino acid sequence of naturally-occurring BPTI and of bovine serum inhibitor (BSI) protein.

Figure 11 is a graphic representation of results of an assay for production of antibody to the alpha/beta peptide complex of the present invention in three rabbits injected with the complex. Pre indicates the control (pre injection) value for an animal; 1st indicates the post injection value for an animal. Animals are indicated by number (276,277,278).

Detailed Description of the Invention

Traditionally it has been thought that fragments of single domain proteins could not fold in aqueous solution and the smallest cooperative unit of protein folding was a domain. The present invention is based on the discovery that short peptides can be joined (e.g., by covalent bonding) to form a peptide complex which interact in much the same manner as the corresponding region in the intact or native protein.

The present invention relates to peptide complexes which are comprised of at least two short peptide units, each of which has an amino acid sequence (primary sequence) corresponding to the amino acid sequence of a selected segment of a protein of interest and which are joined in such a manner that they interact much as the regions in the native or intact protein interact. It also relates to a method of their preparation and a method of their use. Such peptide complexes are useful as synthetic vaccines, as synthetic ligands in raising antibodies to be used in diagnostic tests, as drugs (e.g., hormones, enzymes), in epitope mapping and in designing proteins.

A key element in the development of the peptide complexes of the present invention is the joining of at least two short peptide units by covalent bonding as a means of enhancing the stability of the resulting complex. The amino acid sequences of the peptide units in the complex correspond to the amino acid sequences of regions of defined secondary structure which interact in the native or intact protein. The short peptides units which comprise peptide complexes of the present invention are selected, by the methods described below, on the basis of interactions occurring in the intact protein. The peptide units selected in this manner correspond in sequence to segments in the intact protein which form defined secondary structure (e.g., alpha-helix, beta-sheet). The peptide units may also include sequences which correspond to other segments of the intact protein which enhance the stability of the peptide complex.

The sequence of a peptide unit which corresponds to a selected segment in the intact protein can be identical to that of the selected segment or, alternatively, can include one or more amino acid substitutions. A substitution is by an amino acid which has the same propensity to form the secondary structure observed in the native or intact protein of interest. For example, an alanine or a serine residue can be substituted for the cysteine residue present at residue 55 of the peptide complex represented in Figure 1.

Bonding of the two units will generally be covalent in nature and, in particular, will be disulfide bonding (between at least one cysteine residue on each of the peptide units). Covalent bonding is used because the bond between the short peptide units must be strong enough to hold the units together and overcome their tendency to diffuse away from each other in solution. Alternatively, any covalent bond which does not destroy or alter the conformation can be used (e.g., carboxyl/amino amide linkages, such as a glu/lys link).

Selection of short peptide units to be incorporated into a peptide complex of the present invention can be carried out in at least two different ways: one for those proteins whose crystal structure is known and one for those proteins for which the crystal structure is not known. In both cases, the length of the short peptide units used will be defined or limited by the number of amino acid residues needed to produce stable, non-random structure under physiological conditions.

<u>Selection of short peptide units if protein crystal structure is known</u>

The crystal structure of many proteins is already known and additional structure data can be obtained using known techniques. Leszczynski, J.F. and G.D. Rose, <u>Science</u>, <u>234</u>:849-855 (1986); Brookhaven Protein Data Bank Newsletter, Brookhaven National Laboratory, No. 41, pp. 3-4 New York (7/87). In the case of proteins whose crystal structure is known, selection of short peptide units is carried out in the following manner: The crystal structure of a protein of interest is initially assessed for the occurrence of contacts of less than 4Å between two regions of well-defined secondary structure; such contacts are indicative of favorable interactions between these regions. These regions are then defined and contacts, such as hydrogen bonds between two regions, van der Waals contacts and salt bridges (in which positive and negative side chains interact), are identified. The specific sequences used to make up the short peptide units are determined on the basis of the amino acid residues which make the contacts.

In one embodiment of the present invention, the crystal structure of a protein of interest is assessed for defined areas of contact between protein regions. Once these areas have been defined, the location(s) of disulfide bond(s) is determined, followed by a determination of the occurrence and location of other types of contacts between protein regions.

This approach has been used in assessing the crystal structure of bovine pancreatic trypsin inhibitor (BPTI). This was carried out in the following way: the native crystal structure of BPTI was searched for contacts between/among regions of less than 4Å. In this case, the

occurrence of disulfide bonds was of particular interest. This resulted in identification of two segments of the primary sequence in the intact protein: one which forms an alpha-helix and one which forms a beta-sheet in the native protein. The sequences of the two segments are represented in Figure 1, as are contacts made between the two which are closer than 4Å.

Based on this, the two sequences shown in Figure 1 were determined. One sequence which resulted (sequence 1) includes the 10 amino acid sequence of the alpha-helix (residues 47-56 in Figure 1) and, in addition, a 4 amino acid residue segment (residues 43-46) in which numerous contacts were made with a beta sheet. As shown in Figure 1, a second peptide includes 14 amino acid residues (sequence 2).

Contacts closer than 4Å which occur between these two regions in the intact protein are represented in Figure 1 as hydrophobic "pocket" contacts, secondary structure contacts and disulfide bond contacts.

<u>Selection of short peptide units if protein crystal structure is unknown</u>

In the case of a protein of interest whose crystal structure is unknown, a procedure similar to that described above for selecting short peptide units in proteins of known crystal structure is used.

Assessment of the amino acid sequence of the protein of interest (e.g., of the sequence either as determined, using known techniques and equipment, or as inferred from the nucleotide sequence of DNA encoding the protein) is carried out, in order to identify specific segments in the primary sequence which may have defined secondary structure and to determine the number of cysteine

residues in these regions. By partial proteolysis, which results in generation of numerous shorter fragments of the protein of interest, it is possible to determine if there are disulfide bonds which connect these segments. The occurrence of disulfide bonds connecting these segments is indicative of secondary structure interactions in the intact protein. Short peptide units corresponding to the segments defined in this way can be synthesized, as described below. In those cases in which the protein of interest is not itself available, but the amino acid sequence is known, (e.g., by deduction from the nucleotide sequence which encodes it), it is possible to look at the area(s) in the amino acid sequence in which cysteine residues occur and, thus, where disulfide bonds might also occur.

Construction of short peptide units and peptide complexes

Once segments in which contacts occur have been identified and their amino acid sequences determined, peptide units corresponding in sequence to the amino acid sequence of each of these two regions in the native protein are made. Such peptides can be made mechanically (i.e., on a protein synthesizing machine); can be produced using known genetic engineering techniques, in which DNA encoding each peptide is cloned or synthesized and expressed, followed by recovery of the peptide units; or can be isolated or purified from naturally-occurring sources.

Such peptide units have been made and joined for BPTI. In particular, regions which correspond to the alpha helix and to a substantial fraction of the beta sheet have been produced and joined.

Bovine pancreatic trypsin inhibitor (BPTI) is one of a series of enzymes that inhibit proteolytic activity. Travis, J. and G.A. Salvesen. Rev. Biochem. 52, 655-709 (1982). Such enzymes are important in cell growth and differentiation which requires a series of surface-related proteolytic events involving serine proteinases. BPTI is a single domain protein which contains three disulfide bonds, an alpha helix, a short 3_{10} helix and a 3-stranded beta sheet. The structure of BPTI (58 residues) has been determined in three different crystal forms.

Synthesis of the two short peptide units described above for BPTI is described in detail in Example 1. In the case of BPTI, one short peptide, referred to as alpha peptide, was synthesized mechanically to have the following sequence:

```
 N   N   F   K   S   A   E   D   C   M
Asn-Asn-Phe-Lys-Ser-Ala-Glu-Asp-Cys-Met-
 43  44  45  46  47  48  49  50  51  52

 R   T   A   G   G   A
Arg-Thr-Ala-Gly-Gly-Ala
 53  54  55  56  57  58
```

It includes the C-terminal alpha helix and a short segment of beta sheet. Residues 47-56, inclusive, are those which make up the BPTI alpha-helix. Residues 43-46, inclusive, as described above, make contacts closer than 4Å with residues in the beta peptide, which are referred to as a hydrophobic "pocket". In this peptide an alanine (Ala) residue was substituted at residue 55 for the cysteine (Cys) which occurs at that

location in the native protein. As a result, the alpha peptide used includes only one cysteine residue, rather than two, as is the case with natural BPTI.

The second short peptide, referred to as beta peptide and also synthesized mechanically, has the following sequence:

R	Y	F	Y	N	A	K	A	G	L
Arg-	Tyr-	Phe-	Tyr-	Asn-	Ala-	Lys-	Ala-	Gly-	Leu-
20	21	22	23	24	25	26	27	28	29

C	Q	T	F
Cys-	Gln-	Thr-	Phe
30	31	32	33

This amino acid sequence is the same as that of the native sequence. Disulfide bonding between cysteine residues 30 and 51 was used to join the components to produce the peptide complex, referred to as the alpha/beta complex. In BPTI, the 30-51 disulphide connects the C-terminal α-helical region (containing Cys-51) to a strand of the central antiparallel β-sheet (containing Cys-30). The core region of the alpha/beta complex therefore includes residues corresponding to the α-helix and to a substantial part of the central antiparallel β-sheet. Residues outside this core region were also included. Our objective was to include residues in one peptide that interact with residues in the second peptide (i.e., in the native structure) without allowing the peptides to become too large. The complex represents approximately half of BPTI (30 of 58 residues) and it includes the majority of one of the hydrophobic cores of the protein.

Other forms of bonding can be used to join the two or more peptide units which make up a peptide complex; any covalent bond which does not destroy or alter the conformation can be used.

The synthetic peptide complex of the present invention is very soluble in aqueous solution and does not aggregate. As judged by circular dichroism (CD) and NMR, the peptide complex is over 90% folded in aqueous solution (pH 6) at 4°C. The structure unfolds when the disulphide bond is reduced. Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) indicates that it contains much of the secondary and tertiary structure present in the corresponding region of native BPTI. These results demonstrate that native-like structure can form early in protein folding, and that peptide models can be used to characterize the structures of transient folding intermediates.

Physico-chemical assessment of the characteristics of the individual short peptide units and of the peptide complex

Once the peptide units have been synthesized and joined to produce a peptide complex, many types of measurement can be used to characterize the structure of the peptide complex under physiological conditions. For example, high resolution nuclear magnetic resonance, circular dichroism, ultraviolet absorbance, x-ray crystallography, neutron difraction and other measurements techniques can be used. Use of high resolution nuclear magnetic resonance, circular dichroism and ultraviolet absorbance is described below as they have been applied to the two short peptide units corresponding to the distinct regions in BPTI described and to the

peptide complex they form when joined by a disulfide bond. A similar approach can, however, be used to characterize the structure of any complex.

A. <u>High Resolution Nuclear Magnetic Resonance</u>

High-resolution proton (^{1}H) nuclear magnetic resonance (^{1}H NMR) spectra have been obtained for the BPTI alpha/beta complex of BPTI (described above and in Example 1). Figure 2 is the entire ^{1}H NMR spectrum for the alpha beta complex and Figure 3 is the aromatic chemical shift region of the ^{1}H NMR spectrum temperatures. The spectrum represented in Figure 3 is that for the left-most series of peaks (e.g., from about 6.5 to 7.5 ppm) in Figure 2. In Figure 3, the resonances shown correspond to protons on (the benzene rings found in) the side chains of the aromatic amino acids phenylalanine and tyrosine; phenylalanine occurs twice in peptide sequence 2 and once in sequence 1 and tyrosine occurs twice in the sequence 2, as shown in Figure 1. As also shown in Figure 1, one of the phenylalanine residues and both tyrosine residues present in sequence 2, as well as the phenylalanine present in sequence 1, participate in contacts closer than 4Å in what is referred to as a hydrophobic "pocket." As shown in Figure 3, the frequencies (chemical shift) of some of the resonances change with temperature, while others do not. In particular, the resonance of peak A shows a temperature-dependent chemical shift; that of peak B does not.

The chemical shift of A is the time average of the resonances of at least two rapidly interconverting species whose relative populations change with

temperature. One of these species is probably an unstructured complex while the others are likely to have structure.

Figure 4 shows the chemical shift of peak A at several temperatures. Many factors other than peptide structure might lead to temperature-dependent chemical shifts. However, these factors would typically give rise to a linear temperature dependence. The deviation from linearity depicted in Figure 4 for the chemical shift of peak A can be taken as evidence that the region of the peptide that gives rise to this resonance undergoes a thermal transition at about 40°C. It is likely that this thermal transition results from the loss of structure in alpha/beta complex as the temperature is raised above 40°C. This indicates the presence of structured alpha/beta complex at temperatures below 40°C.

Two dimensional NMR spectroscopy (2D-NMR) was used in order to further characterize the structure in the alpha/beta complex. Ninety percent of the resonances in spectra of the alpha/beta complex have been assigned using sequential strategies. Wuthrich, K., NMR of Proteins and Nucleic Acids, Wiley-Intersicence, New York (1986). Unequivocal starting assignments were obtained as described in Example 4.

Although the NMR assignments for the alpha/beta complex were made independently of the assignments for BPTI, the relative chemical shifts in the two species are remarkably similar (Figure 5). For comparison, the range of chemical shifts for amino acid residues in model "random-coil" peptides, Bundi, A. and Wuthrich, K., Biopolymers, 18:285-297 (1979), are indicated with squares in Figure 5. These results suggest that the

structures in the complex and native BPTI are similar, and that the population of folded alpha/beta complex molecules at 4°C is high.

Analysis of the two dimensional nuclear Overhauser effect spectroscopy (NOESY) data indicates that most, if not all, of the secondary structure present in the corresponding region of BPTI is also present in the folded conformation of the alpha/beta complex. In particular, the C-terminal α-helix and the central anti-parallel β-sheet appear to be intact (Table I). Tertiary interactions between these regions of secondary structure are also present in the alpha/beta complex (Table I). These tertiary interactions are similar to those found in intact BPTI.

Table I Selected NOE's observed in NMR spectra of PαPβ.

Structure	Observed NOESY cross-peak	Distance in crystal structure of BPTI
β-sheet	21α - 32α	2.4Å
	23α - 30N	4.4Å
	24α - 29N	3.4Å
	26α - 27N	3.5Å
α-helix	47β - 49N	2.6Å
	48β - 52N	4.7Å
	52α - 55β	4.2Å
	54N - 55N	2.6Å
tertiary	21δ - 46α	4.1Å
	21ε - 48N	3.2Å
	23ε - 55β	4.1Å
	23β - 55β	2.5Å
	30β - 48β	3.5Å
	30N - 52γ	3.4Å
	45δ - 50α	4.8Å
	45ε - 54γ	2.8Å
	45ε - 55β	4.5Å

All listed NOE's were observed as cross-peaks which could be unambiguously assigned (i.e., definitive assignments of resonances to the associated protons, and no possibility of overlap with other resonances) in NOESY spectra of the alpha/beta complex at 4°C. Cross-peaks were observed both in spectra accumulated using 250 msec and 350 msec mixing times. For comparison, the distances

between the corresponding protons in the crystal structure of BPTI are indicated. Wlodawer, A. et al., J. Mol. Biol., 180:301-329 (1984).

B. Circular Dichroism

Circular dichroism (CD) is useful in measuring the overall helix content of peptides in solution. The n ⟶ * absorbance band centered at 222 nM is due to the amide bonds found in the backbone of the peptide. The CD ellipticity of this band has been used as an indicator of the alpha helix content of peptides and proteins. Schellman, J.A. and Becktel, W.J., Biopolymers, 22:171 (1983) and Holzwarth, G. and Doty, P. Journal of the American Chemical Society, 87:218 (1965). CD was used, as described in Example 2, to determine whether the alpha/beta complex has a more stable structure than is present in either or both of the individual peptide units. Results of this assessment are represented in Figures 6 and 7.

Figure 6 shows the CD spectra of the alpha peptide unit at 0 and 60°C (5A), the beta peptide unit at 5°C (5B) and the alpha/beta peptide complex at 0, 10, 40 and 60°C (5C). The spectra of the alpha and beta peptides do not show evidence of stable structure for these peptides, even at low temperatures where such structure would be favored. However, the spectra of alpha/beta complex at low temperatures shown in Figure 5C do show evidence for the presence of alpha helical structure as indicated by the negative ellipticity near 222 nM and the ratio of ellipticities at 222 and 208 nM. These criteria have been used previously to detect the presence of hellicity in peptides. Shoemaker, K.R. et al., "Circular Dichroism

Measurement of Peptide Helix Unfolding", Tenth Peptide Symposium, June 1987.

Additional evidence for the presence of stable structure in alpha/beta complex and the lack of it in either alpha or beta peptide alone is shown in Figure 7. Figures 7A, 7B and 7C show the CD ellipticity at 222 nM for peptides alpha, beta and alpha/beta complex, respectively, at various temperatures. As with NMR chemical shifts, a non-linear temperature dependence of this parameter can be taken as evidence for a thermal transition, (e.g., change in structure). As seen in Figure 7B, the temperature dependence of the beta peptide CD signal is completely linear, indicating the absence of a thermal transition and therefore probable lack of structure at any temperature in this peptide.

The data for the alpha peptide (Figure 7A) show a possible thermal transition between 10 and 20^{o}C. On this basis, the peptide may have weak helicity at low temperatures. However, because of the low transition temperature, it is unlikely that the alpha peptide would exhibit significant amounts of structure at physiological temperatures (i.e., 37^{o}C). In contrast, the data shown in Figure 6C for alpha/beta complex indicates a thermal transition at a temperature around 40^{o}C, consistent with the apparent transition temperature seen in the temperature dependence of the chemical shift of peak A in the NMR spectrum (See Figure 4). This indicates that alpha/beta complex has much more stable non-random structure than either of its component peptides and that there is likely to be significant amounts of structure present in this complex under physiological conditions.

CD spectras indicate that the alpha/beta complex folds into a native-like conformation in aqueous solution and that this structure can be unfolded with increasing temperature, whereas there is no evidence for substantial folded structure when the disulphide bond is reduced. As monitored by CD, the thermal unfolding transition for the complex is broad, spanning over 60°C, and a fully folded baseline is not reached even at 0°C (Figure 8). The transition is completely reversible up to 80°C provided that the sample is degassed before use, and can be eliminated by adding the denaturant, GuHCl. The complex does not aggregate, as judged by gel filtration, and there is no significant dependence of the CD signal on peptide concentration over a 20-fold range.

C. Ultraviolet Absorbance

In Figure 9 the ultraviolet absorbance due to tyrosine, at a wavelength of 285.5 nM, and at a variety of temperatures, is represented. The temperature dependence of tyrosine absorbance has been used to monitor the thermal unfolding of proteins. Atlas of Protein Spectra in the Ultraviolet and Visible Regions, Ed: D.M. Kirschenbaum, IFI/Plenum, New York (1972). Again, non-linear dependence of this parameter on temperature is indicative of a thermal transition. As shown in Figure 9, a deviation from linearity occurs at about 40°C for tyrosine absorbance, indicating a thermal transition at this temperature. This is agreement with the CD and NMR results and again indicates the presence of stable structure in alpha/beta complex at physiological temperatures.

Summary

The analysis, using the three types of measurement described above, is support for the fact that:

1. the individual short peptide units (i.e., the alpha peptide sequence and the beta peptide sequence) do not display secondary structure formation at physiological temperatures;

2. the alpha/beta complex does exhibit a thermal transition, as evidenced by the deviations from linearity in temperature dependence evidenced by the complex in all three measurements; and

3. the presence of a thermal transition at 40°C, above the common physiological temperature of 37°C, indicates that alpha/beta complex possesses significant amounts of stable, non-random structure under physiological conditions.

These conclusions are significant because the relative population of structured peptide complex is critical to its ability to cause a strong immune response and the production of antibodies which are strongly cross-reactive to the native protein. It is this property, i.e., the presence of significant amounts of structure at physiological temperatures, which is lacking in peptide antigens available by present methods.

A similar analysis, in which deviation from linearity of temperature dependence (i.e., nonlinear temperature dependence) is a key criterion for or indication of stable structure formation by a peptide complex comprised of at least two short peptide units whose sequences correspond to the amino acid sequence of at least two regions in a protein of interest, as

described above, can be used to assess and verify the stability of any peptide complex.

Thus, using the approach described above, regions of a protein of interest which are involved in (responsible for) folding of the protein and, thus, for stable conformation of the protein, can be identified for any protein of interest. Short peptide units corresponding in amino acid sequence to the selected regions can be synthesized, using known techniques. At least two of the short peptide units are then joined, preferably by covalent bonding, which can be disulfide bonding, to produce a peptide complex. The resulting complex will be more stable in aqueous solution than individual short peptides. The conformation of the peptide complex can be assessed, if necessary, by one or more of the three methods described for assessment of stable structure formation of the BPTI peptide complex.

The present invention will now be illustrated by the following examples, which are not intended to be limiting in any way.

EXAMPLE 1 Peptide Complex Formation

Peptides alpha and beta were synthesized on an Applied Biosystems Model 430A peptide synthesizer using standard reaction cycles (std-1r). Applied Biosystems Peptide Synthesizer 430A User's Manual, Version 1.2 (1985). All amino acids were alpha-amino protected with t-BOC and were coupled as symmetric anhydrides, except asparagine, glutamine and arginine, which were coupled as HOBt esters. The side chain protecting groups were as follows: arginine, MTS; methionine, sulfoxide; cysteine, 4-methoxy O-benzyl; lysine, 2 Cl-Z; tyrosine, Br-Z;

serine and threonine, benzyl; glutamate, O-benzyl. For the synthesis of alpha, PAM-alanine resin was used. For beta synthesis, PAM-phenylalanine resin was used. Ninhydrin measurements were made on every coupling cycle and multiple couplings were used to produce coupling efficiencies of at least 98%. Both peptide-resins were cleaved using the "Low-High" TFMSA cleavage procedure. User Bulletin No. 16, Peptide Synthesizer, Applied Biosystems (1986). The crude peptide products were purified on Sephadex G-15 in 5% Acetic Acid/H_2O. The elution of each peptide was monitored by A_{234} and was found to elute in the void volume. The G-15 purified peptides were lyophilized and stored dessicated at -20°C.

<u>Alpha/beta complex formation</u>

Alpha/beta complex was produced using G-15 purified alpha and beta. The oxidation of the disulfide bond was carried out in 0.1 <u>M</u> Tris, 0.2 <u>M</u> KCl at pH 8.7, using peptide concentrations of approximately 20 m<u>M</u>. Creighton, T.E., <u>Journal of Molecular Biology</u>, <u>113</u>:275-293 (1977). The oxidation reaction was catalyzed in air by vigorous stirring for 30 h at 4°C. The crude reaction mixture was lyophilized and stored dessicated at -20°C.

Alpha/beta complex was also produced by the following method: Beta peptide was reacted with oxidized glutathione (GSSG) and the mixed disulfide was purified by HPLC, to produce P-beta SSG. P-beta SSG was then reacted with P alpha to produce P alpha P beta.

Alpha, beta and alpha/beta complex HPLC purification

Each of the peptides studied were purified by semi-preparative reverse phase HPLC using a C-18 (1 x 20 cm) column (Vydac). The peptides were eluted in 0.1% TFA in H_2O/0.1% TFA, 30% CH_3CN in H_2O linear gradients optimized for each peptide. The elution was monitored by A_{229} and fractions were collected and lyophilized. The purity of each peptide, as assessed by analytical reverse phase HPLC was greater than 99%.

The composition of alpha beta complex was confirmed by reduction of the disulfide bond in 10m*M* DTT, 0.1*M* Tris, pH 7.0, followed by analytical reverse phase HPLC to produce peaks with retention times identical to those of alpha and beta. The sequences of alpha and beta were confirmed using an Applied Biosystems Model 470A gas-phase sequencer.

EXAMPLE 2 Homology of the alpha/beta complex of BPTI to protease inhibitor sequence within amyloid Beta-protein precursor

This Example illustrates that the amino acid sequence of the alpha/beta complex synthesized in this invention demonstrates homology with the protein domain containing a protease-inhibitor sequence, within the precursor of the amyloid Beta-protein characteristics of Alzheimer's disease.

Amyloid Beta-protein/amyloid A4 has been shown to be a peptide present in the neuritic plaques, neurofibrillary tangles and cerebrovascular deposits in individuals with Alzheimer's disease or Down's Syndrome (trisomy 21). It may be involved in the pathogenesis of Alzheimer's disease. Tanzi, R.E. et al., Nature, 331:528-530 (1988).

Amyloid protein has recently been shown to be encoded as part of a larger protein (amyloid protein precursor) by a gene referred to as the amyloid protein precursor (APP) gene. Ponte, P. et al., Nature 331, 525-527 (1988); Tanzi, R.E. et al., Nature, 331 528-530 (1988); Kitaguchi, N. et al., Nature, 331 530-532 (1988);

Figure 10 compares the amyloid precursor protein (APP) domain with the BPTI alpha/beta complex of the present invention. Also shown are residues of naturally-ocurring bovine pancreatic typsin inhibitor precursor protein and bovine serum inhibitor protein (BSI). The sequence of the peptides of alpha/beta complex of BPTI are shown in Figure 10 (see underlining of the BPTI sequence and of the homologous sequences of the amyloid precursor protein).

Numbering is based on the predicted amyloid protein sequence as defined in Ponte, P. et al., and Kitaguchi et al., see id.

Residues homologous to the highly conserved sequences of the basic typsin inhibitor family, which includes BPTI, bovine inter-alpha typsin inhibitor, human inter-alpha typsin inhibitor, sea anemone proteinase inhibitor, and Russell's viper venom basic protease inhibitor (Kitaguchi, N., et al id) are shown in Figure 8 by the dark overlining. The basic amino acid residue (arginine) in the active site is indicated by an asterisk.

The alpha and beta sequences align with the amyloid precursor protein. Of the 56 residues shown in APP, 13 residues of the alpha/beta complex (23%) are identical.

EXAMPLE 3 Use of synthetic BPTI sub-domain as immunogenic peptides

Antibody production in three rabbits injected with the alpha/beta BPTI peptide complex of the present invention was assessed in the following manner: Prior to injection, animals were bled to provide pre-injection (baseline) values. Animals were subsequently injected with the alpha/beta complex as described previously; the complex was administered in an adjuvant in a series of doses. Animals were subsequently bled and their sera assayed, using an ELISA technique, for alpha/beta antibody production (binding of native BPTI to antibodies raised against the alpha/beta complex). Results (pre- and post-injection) are presented in the Table and represented graphically in Figure 11. In one of the three rabbits (278), the results clearly demonstrated that the complex is an effective antigen.

THE TABLE O.D. at 410 NM

	LOG DIL	PRE 276	1ST 276	PRE 277	1ST 277	PRE 278	1ST 278
1	CONC.	0.058	0.083	0.130	0.152	0.041	1.079
2	1	0.053	0.078	0.071	0.082	0.035	1.079
3	2	0.024	0.029	0.018	0.039	0.028	0.960
4	3	0.001	0.001	-0.001	0.002	-0.002	0.283
5	4	-0.006	-0.009	-0.008	-0.009	-0.009	0.035

EXAMPLE 4 Characterization of the structure of Alpha, Beta and Alpha/Beta Complex High Resolution Nuclear Magnetic Resonance (^1H NMR)

^1H NMR spectra were obtained on a home-built NMR Spectrometer at 500 MHZ (National Magnet Laboratory, M.I.T.). Purified alpha/beta complex was pre-exchanged with D_2O, lyophilized and taken up in 99.995% D_2O to a concentration of 27 mg/ml. The pH of the resulting solution was approximately 5.0. The ^1H NMR spectra were collected using a simple one-pulse sequence with a recycle delay of 3 seconds. Approximately 80 scans were collected for each spectrum. Chemical shifts were referenced to an internal standard of TMSP (Trimethylsilylpropianate). Temperature was maintained to precision of $\pm .1^oC$.

Circular Dichroism Spectra

The CD spectra of alpha, beta and alpha-beta complex were obtained on an AVIV 60H CD spectrophotometer. Temperature was controlled using an HP 89100A thermoelectric temperature controller. Spectra were collected at various temperatures and wavelengths and were corrected by subtraction of a buffer blank collected at each temperature. The concentration of peptide was 0.1 mg/ml in a buffers consisting of 5mM potassium phosphate, 0.1M potassium fluoride at pH 6.7 or 0.2 M sodium sulfate, 10 mM disodium hydrogen phosphate at pH 6.0. Spectra of alpha and beta were collected in the presence of DTT.

Two Dimensional NMR Spectroscopy

Double-quantum COSY, RELAY, TOCSY and phase-sensitive NOESY spectra (800 t_1 X 2048 t_2) were collected on a 500 MHz Bruker spectrometer at the Fox Chase Medical Center (Philadelphia, PA) with a recycle delay of 2.5 sec. The mixing times for the NOESY spectra were 250 or 350 msec. Peptide concentration was 15 mM. A sweep width of 5 kHz was used in both dimensions, and a phase-shifted sine-bell function was used to enhance resolution. Definitive assignments that served as starting points for the sequential assignments were made as follows: The methyl resonances of Ala-25 and Ala-48 were assigned using alpha/beta complex variants which contained deuterated alanine (introduced by peptide synthesis) at those sites. The methyl resonances of the only leucine, Leu-29, were assigned on the basis of the unique COSY pattern. The methyl resonances of Thr-54 and Thr-32, which are the only threonine residues found in alpha and beta peptides, respectively, were assigned first in spectra of the isolated peptides at high temperatures (60°C) on the basis of their COSY patterns and intrinsic chemical shift values. These assignments were then transferred to spectra of alpha/beta complex at 60°C (i.e., unfolding conditions) and the resonances were followed as a function of temperatures to 4°C. From these starting points, sequential assignment strategies were used. Wuthrich, K., supra.

Ultra-violet Absorbance Spectra

The ultra-violet absorbance spectrum from 325 to 250 nM of alpha beta complex was obtained at various

temperatures on an AVIV Model 118DS spectrophotometer. Alpha-beta complex was dissolved in the same buffer used for CD spectra to a concentration of 3 mg/ml. The difference in absorbance at 285.5 nM between the spectrum obtained at various temperatures and that obtained at 2°C was calculated using spectral subtraction.

Utility

Peptide complexes and methods disclosed herein are useful in creating peptide complexes, which include at least two short peptide units, each of which corresponds to the amino acid sequence of selected regions of a protein of interest and which are joined in such a manner that the peptide units interact in much the same way that the regions of the intact or native protein which they "mimic" interact. As a result, the peptide complexes of the present invention also mimic the function or activity of the corresponding region of the intact or native protein. As a result, they can be used, as described herein, for production of antibodies directed against them. Synthetic ligands produced according to this invention are especially useful in investigating protein binding sites. In addition, antibodies raised against the alpha/beta BPTI can be used for detection of Alzheimer's amyloid protein and, thus, can be used in diagnostic and immunotherapeutic reagents directed against this protein.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the

invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

1. A method of making a peptide complex comprising covalently bonding at least two short peptide units, each of which corresponds in amino acid sequence to an amino acid sequence in an intact protein of interest which forms defined secondary structure in the intact protein of interest.

2. A peptide complex produced by the method of Claim 1.

3. A method of Claim 1 wherein each short peptide unit is from approximately five amino acid residues to approximately 30 amino acid residues, inclusive, in length.

4. A peptide complex produced by the method of Claim 3.

5. A method of Claim 1 wherein the covalent bonding is disulfide bonding.

6. A peptide complex produced by the method of Claim 5.

7. A method of making a peptide complex which comprises at least two short peptide units, the amino acid sequence of each short peptide unit corresponding to the amino acid sequence of a segment of an intact protein of interest, and which has stable structure in aqueous solution, the method comprising joining at least a first short peptide unit which corresponds to a first segment in the protein of interest which folds into an alpha-helix in the

intact protein of interest and a second short peptide unit which corresponds to a second segment in the protein of interest which folds into a beta-sheet in the intact protein of interest, the first region and the second region interacting with each other in the intact protein of interest.

8. A peptide complex produced by the method of Claim 7.

9. A method of Claim 7 wherein the first short peptide and the second short peptide is each approximately from five amino acid residues to 30 amino acid residues, inclusive, in length.

10. A peptide complex produced by the method of Claim 9.

11. A method of Claim 9 wherein the short peptide units are joined by covalent bonding.

12. A peptide complex produced by the method of Claim 11.

13. A method of making a peptide complex which, in aqueous solution, has a stable structure similar to the structure of regions of a protein of interest, the method comprising:

a. selecting at least a first short peptide unit and a second short peptide unit, the first short peptide unit having an amino acid sequence corresponding to the amino acid sequence of a first region of the protein of interest and the second short peptide unit having an amino acid sequence

corresponding to the amino acid sequence of a second region of the protein of interest, the first region and the second region interacting with one another in the protein of interest, and

b. joining the first short peptide unit and the second short peptide unit by at least one covalent bond such that the two peptide units interact with one another in a manner similar to the interaction between the first region and the second region in the protein of interest.

14. A method of making a peptide complex comprising at least two peptide units, which forms a stable structure in aqueous solution, comprising joining:

a. a first peptide unit of from approximately five to approximately 30 amino acid residues, inclusive, the sequence of which includes at least one cysteine residue and corresponds to the amino acid sequence of a first region of defined secondary structure of a protein of interest and

b. a second peptide unit of from approximately five to approximately 30 amino acid residues, inclusive, the sequence of which includes at least one cysteine residue and corresponds to the amino acid sequence of a second region of defined secondary structure of the same protein of interest as in a),

the first peptide unit and the second peptide unit being joined by at least one covalent disulfide bond between the cysteine residue in the first peptide unit and the cysteine residue in the second peptide unit.

15. A peptide complex produced by the method of Claim 14.

16. A method of making a peptide complex comprising at least two short peptide units, comprising joining by disulfide bonding:

a. a first peptide unit of approximately 14 amino acid residues, the amino acid sequence of the first peptide unit corresponding to the amino acid sequence of a segment of bovine pancreatic trypsin inhibitor which includes at least one cysteine residue and which folds into an alpha-helix in bovine pancreatic trypsin inhibitor and

b. a second peptide unit of approximately 17 amino acid residues, the amino acid sequence of the second peptide unit corresponding to the amino acid sequence of a segment of bovine pancreatic trypsin inhibitor which includes at least one cysteine residue and which folds into a beta-sheet in bovine pancreatic trypsin inhibitor.

17. A peptide complex produced by the method of Claim 16.

18. A peptide complex comprising at least two short peptide units:

a) a first short peptide unit whose amino acid sequence corresponds to the amino acid sequence of a first segment of a protein of interest which forms defined secondary structure in the protein of interest and

b) a second short peptide unit whose amino acid sequence corresponds to the amino acid sequence of a second segment of the same protein of interest as in a) which forms defined secondary structure in the protein of interest,

the first short peptide unit and the second short peptide unit joined by covalent bonding.

19. A peptide complex of Claim 18 wherein the two short peptide units are each from approximately five amino acid residues to approximately 30 amino acid residues, inclusive, in length and each includes at least one cysteine residue and the covalent bonding is disulfide bonding between a cysteine residue in the first short peptide unit and a cysteine residue in the second short peptide unit.

20. A peptide complex of Claim 18 wherein the amino acid sequence of the first short peptide additionally comprises amino acids corresponding to the amino acid sequence of a third segment of the intact protein which enhances the stability of the peptide complex, the amino acid sequence of the second short peptide additionally comprises amino acids corresponding to the amino acid sequence of a fourth segment of the intact protein which enhances the stability of the peptide complex, alone or in combination.

21. A peptide complex comprising:
a. a first peptide, of from approximately five to approximately 30 amino acid residues, inclusive, in length, having an amino acid sequence corresponding to the amino acid sequence of a segment of a protein of interest which forms an alpha-helix or a beta-sheet in the protein of interest and
b. a second peptide, of from approximately five to approximately 30 amino acids, inclusive, in length, having an amino acid sequence corresponding to the amino acid sequence of a segment of the same protein of interest as in a) which forms an alpha-helix or a beta-sheet in the protein of interest,
the first peptide and the second peptide being joined by at least one covalent bond.

22. A peptide complex comprising:
a. a first peptide, approximately 14 amino acid residues in length, having an amino acid sequence corresponding to the amino acid sequence of a selected segment of bovine pancreatic trypsin inhibitor which forms an alpha-helix in bovine pancreatic trypsin inhibitor and which includes at least one cysteine residue and
b. a second peptide, approximately 17 amino acid residues in length, having an amino acid sequence corresponding to the amino acid sequence of a selected segment of bovine pancreatic trypsin inhibitor which forms a beta-sheet in bovine pancreatic trypsin inhibitor and which includes at least one cysteine residue,
the first peptide and the second peptide joined by a disulfide bond.

23. A peptide complex comprising:

a. a first peptide unit having the following amino acid sequence:

Asn-Asn-Phe-Lys-Ser-Ala-Alu-Asp-Cys-Met-Arg-Thr-Ala-Gly-Gly-Ala and

b. a second peptide unit having the following amino acid sequence:

Arg-Tyr-Phe-Tyr-Asn-Ala-Lys-Ala-Gly-Leu-Cys-Gln-Thr-Phe,

the first peptide unit and the second peptide unit being joined by a disulfide bond.

24. A peptide complex comprising at least the following:

a. a first peptide unit of from 5 to 30 amino acid residues, inclusive, the amino acid sequence of the unit corresponding to the amino acid sequence of a first segment of an intact protein of interest, the segment including a first portion which forms defined secondary structure in the intact protein and a second portion which enhances the stability of the peptide complex and

b. a second peptide unit of from 5 to 30 amino acid residues, inclusive, the amino acid sequence of the unit corresponding to the amino acid sequence of a second segment of the intact protein of interest which forms defined secondary structure in the intact protein,

the first peptide unit and the second peptide unit covalently bonded.

25. A peptide complex of Claim 24 wherein the second peptide unit additionally comprises an amino acid sequence corresponding to the amino acid sequence of a portion of the protein of interest which enhances the stability of the peptide complex.

26. A vaccine which is a peptide complex comprising at least two short peptide units, each of which corresponds in amino acid sequence to the amino acid sequence to the amino acid sequence of a segment of an intact protein of interest which forms defined secondary structure, the short peptide units joined by covalent bonding.

27. An antibody capable of binding the peptide complex of Claim 23.

28. An antibody capable of binding the protease inhibitor domain of the amyloid protein precursor.

29. A method of detecting amyloid protein precursor in a biological sample, comprising contacting the sample with an antibody capable of binding the peptide complex of Claim 23, under conditions appropriate for binding of the antibody and amyloid protein precursor to occur.

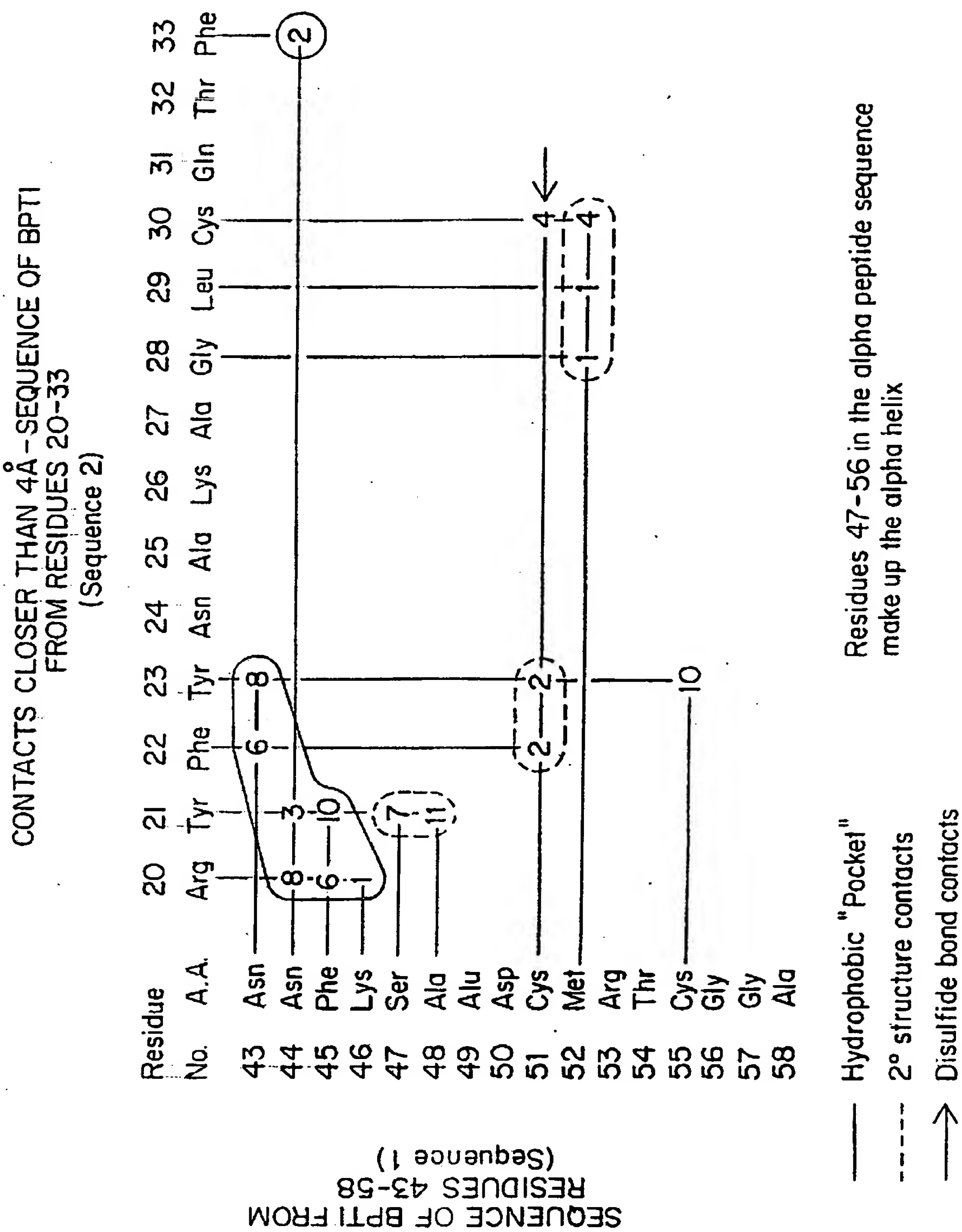

CONTACTS CLOSER THAN 4Å -SEQUENCE OF BPTI
FROM RESIDUES 20-33
(Sequence 2)
20 21 22 23 24 25 26 27 28 29 30 31 32 33
Arg Tyr Phe Tyr Asn Ala Lys Ala Gly Leu Cys Gln Thr Phe
Residue
No. A.A.
43 Asn
44 Asn
45 Phe
46 Lys
47 Ser
48 Ala
49 Alu
50 Asp
51 Cys
52 Met
53 Arg
54 Thr
55 Cys
56 Gly
57 Gly
58 Ala
SEQUENCE OF BPTI FROM
RESIDUES 43-58
(Sequence 1)
Residues 47-56 in the alpha peptide sequence
make up the alpha helix
Hydrophobic "Pocket"
2° structure contacts
Disulfide bond contacts

Fig. 1

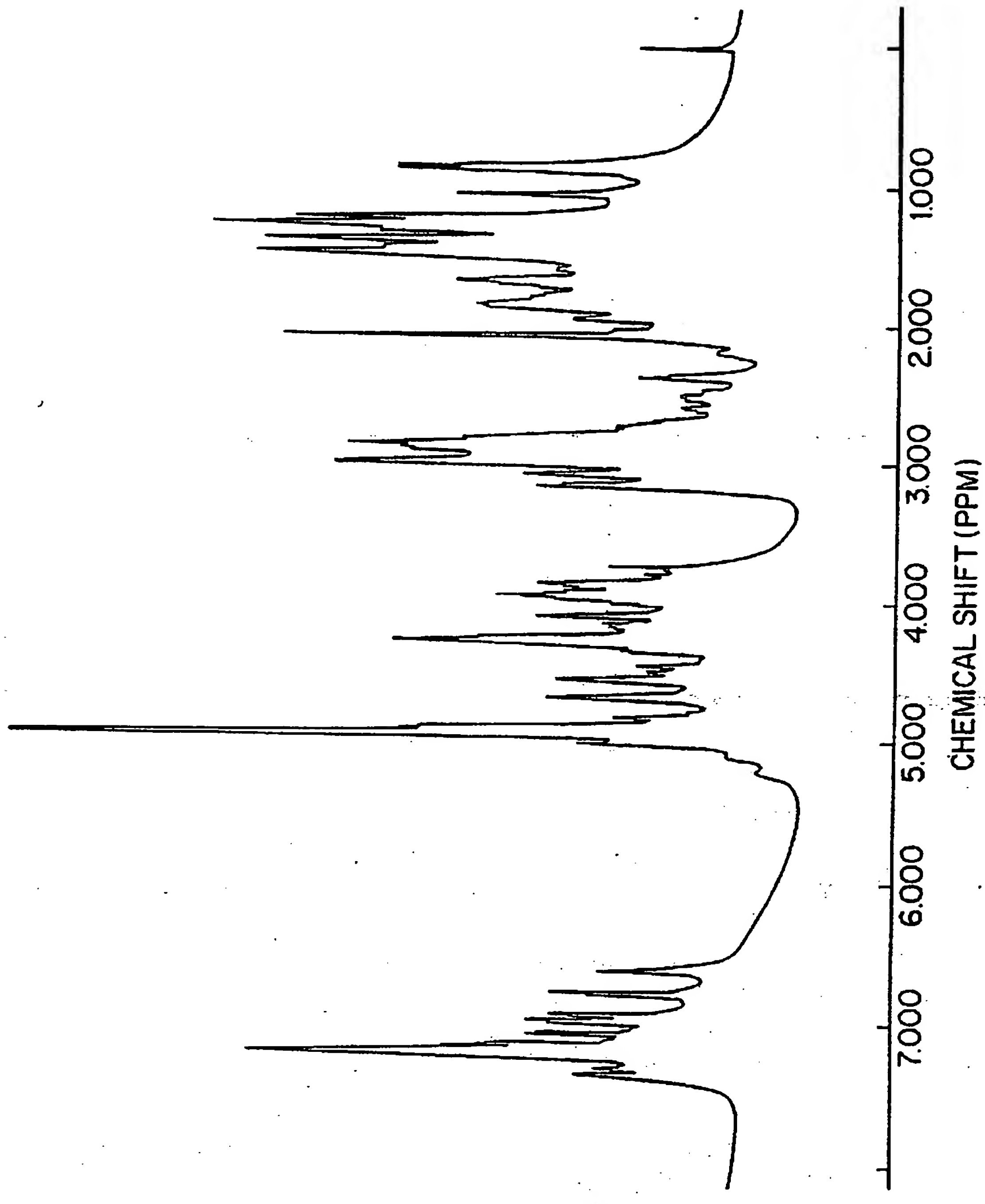


Fig. 2

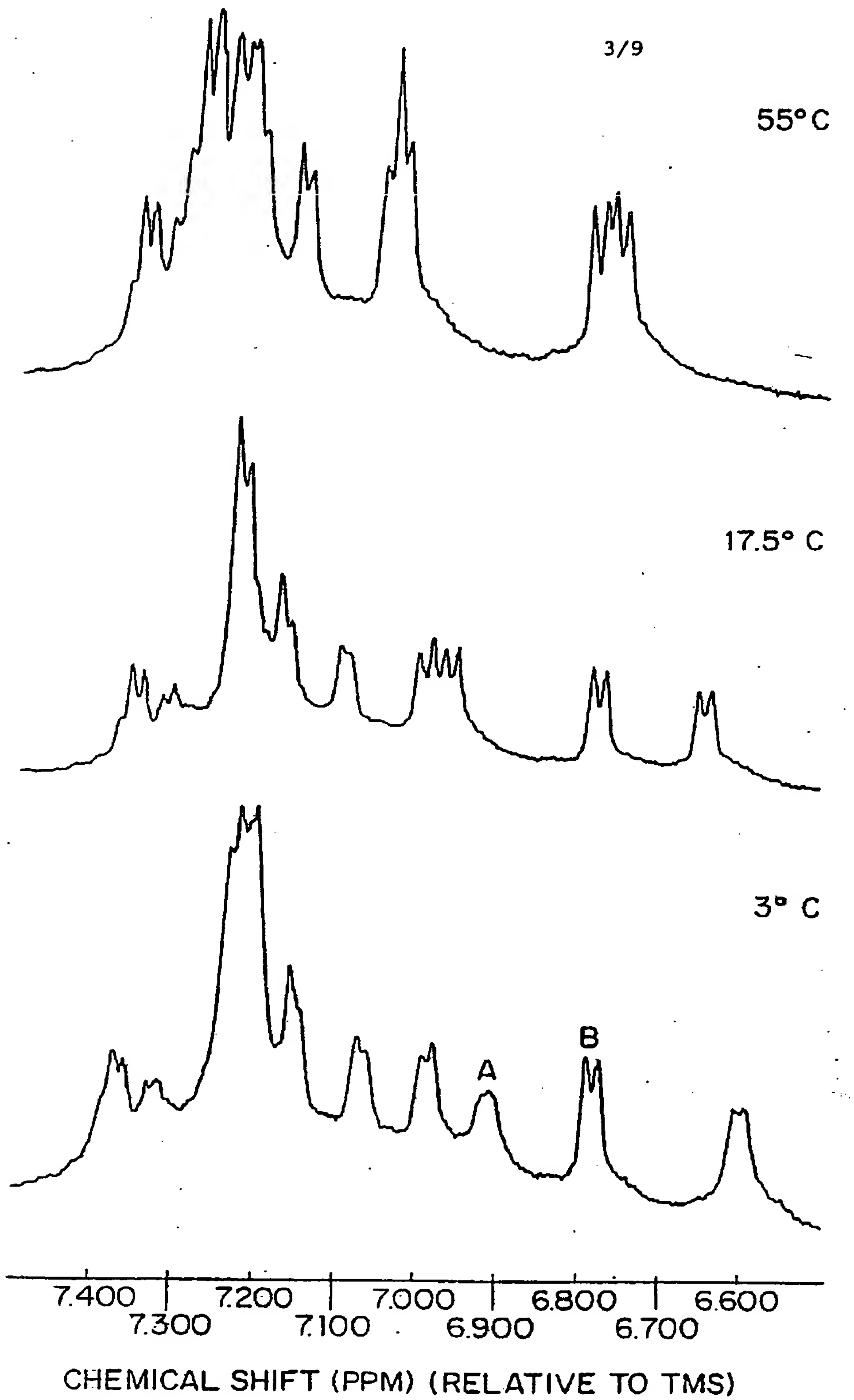

55°C
17.5° C
3° C
A
B
7.400
7.300
7.200
7.100
7.000
6.900
6.800
6.700
6.600
CHEMICAL SHIFT (PPM) (RELATIVE TO TMS)

Fig. 3

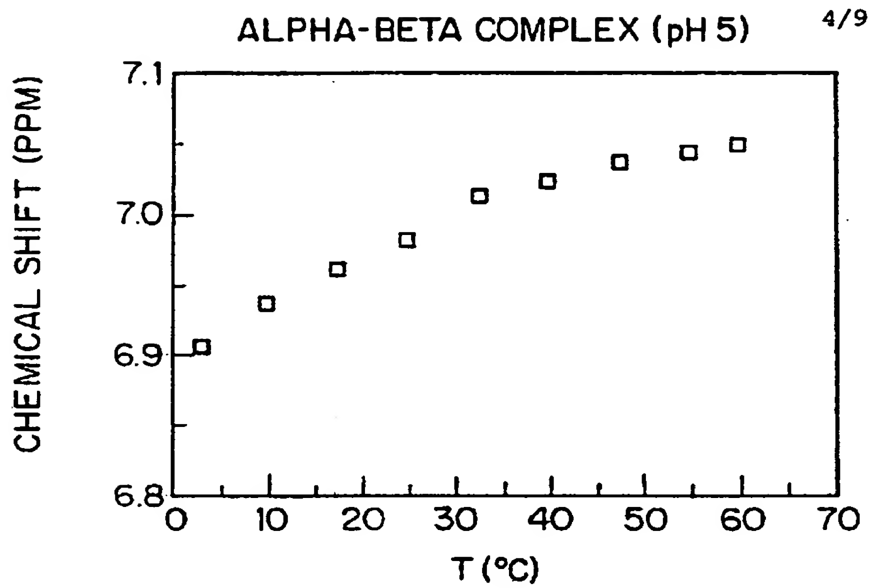

ALPHA-BETA COMPLEX (pH 5)
CHEMICAL SHIFT (PPM)
7.1
7.0
6.9
6.8
0
10
20
30
40
50
60
70
T (°C)

Fig. 4

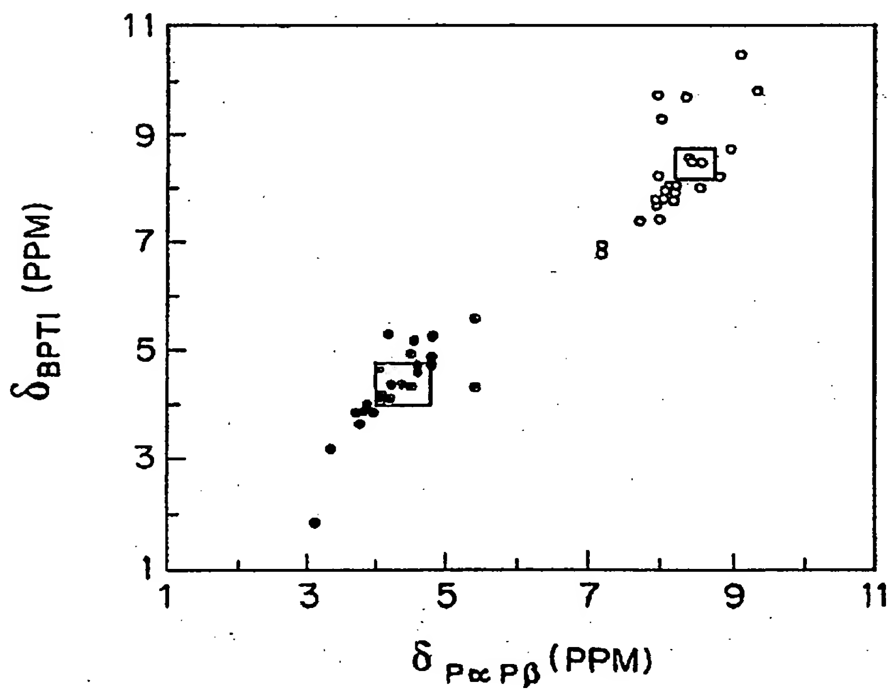

11
9
7
5
3
1
δ_{BPTI} (PPM)
1
3
5
7
9
11
$\delta_{P\alpha P\beta}$ (PPM)

Fig. 5

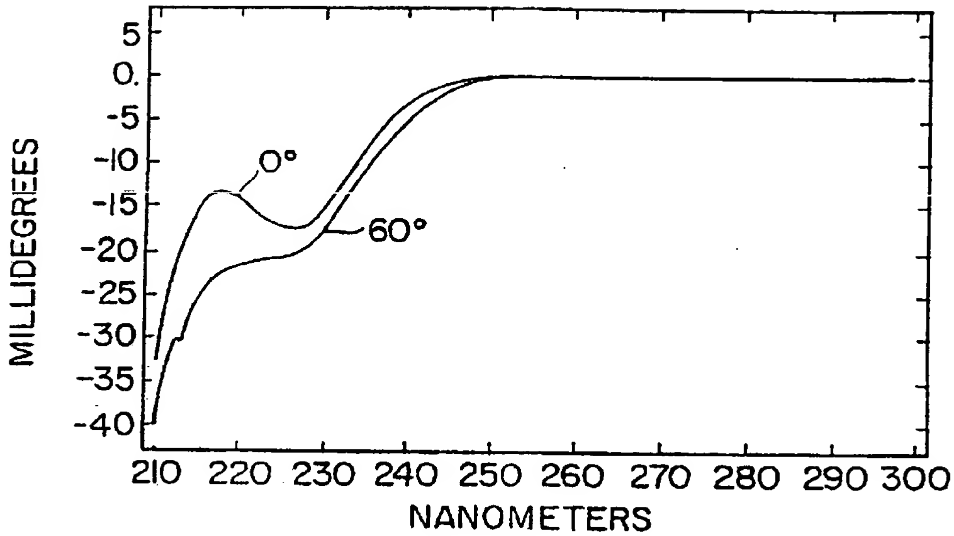


Fig. 6A

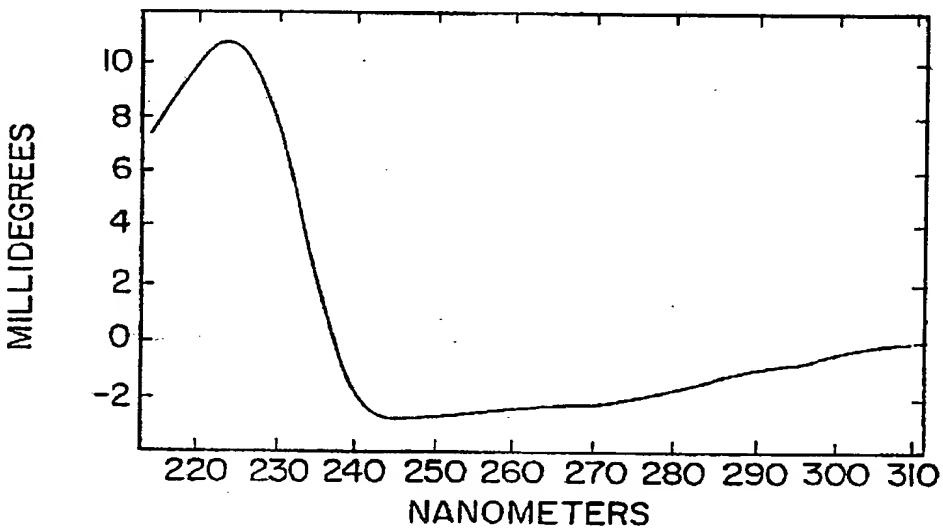


Fig. 6B

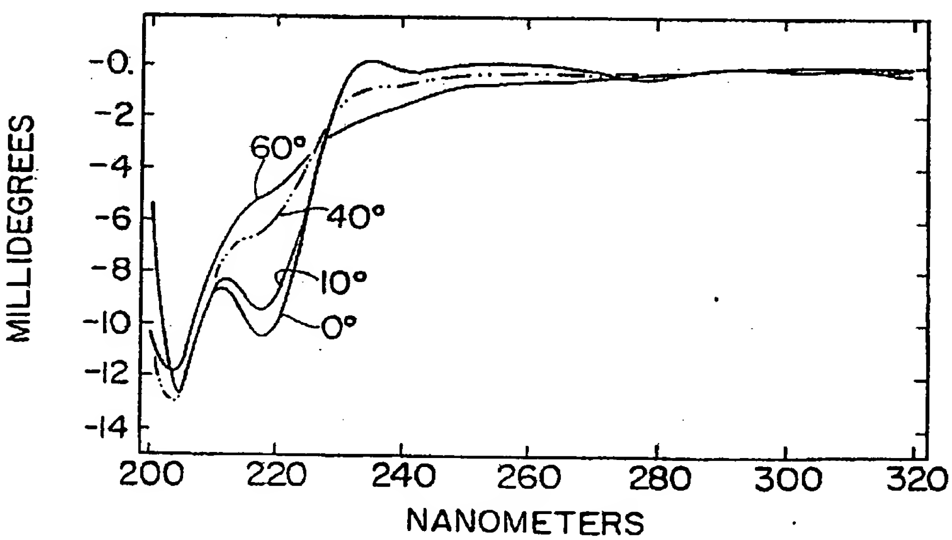


Fig. 6C

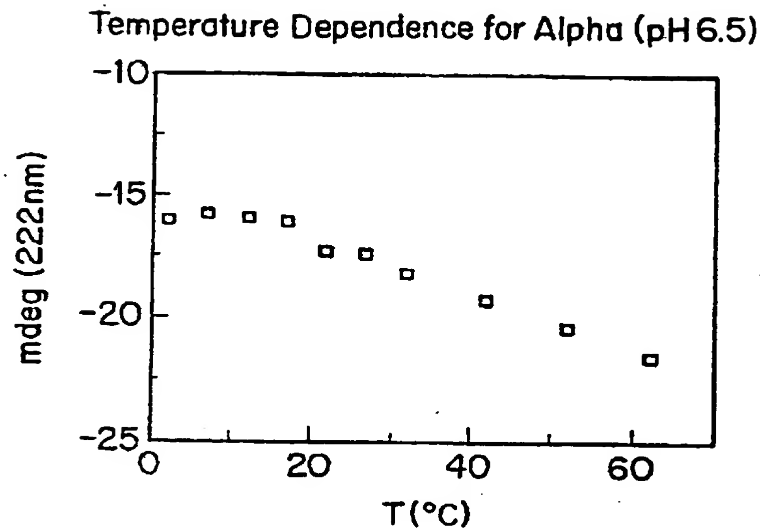


Fig. 7A

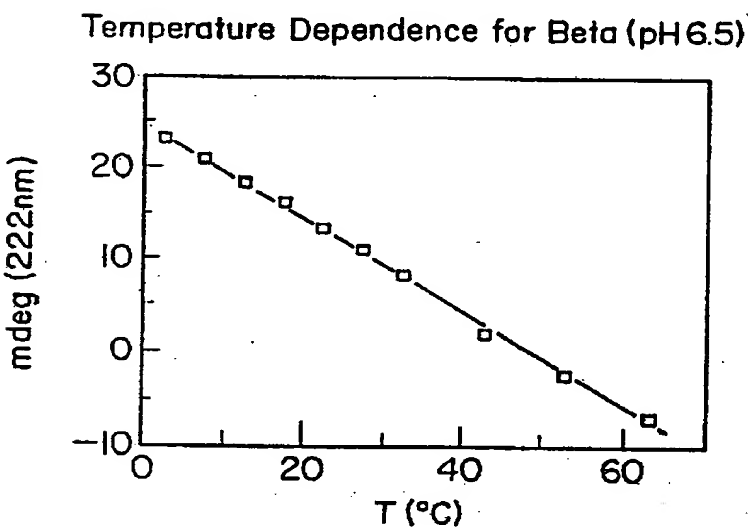


Fig. 7B

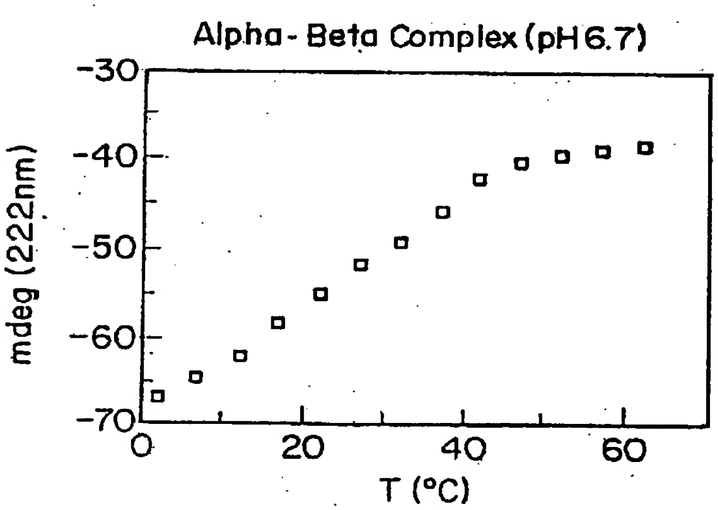


Fig. 7C

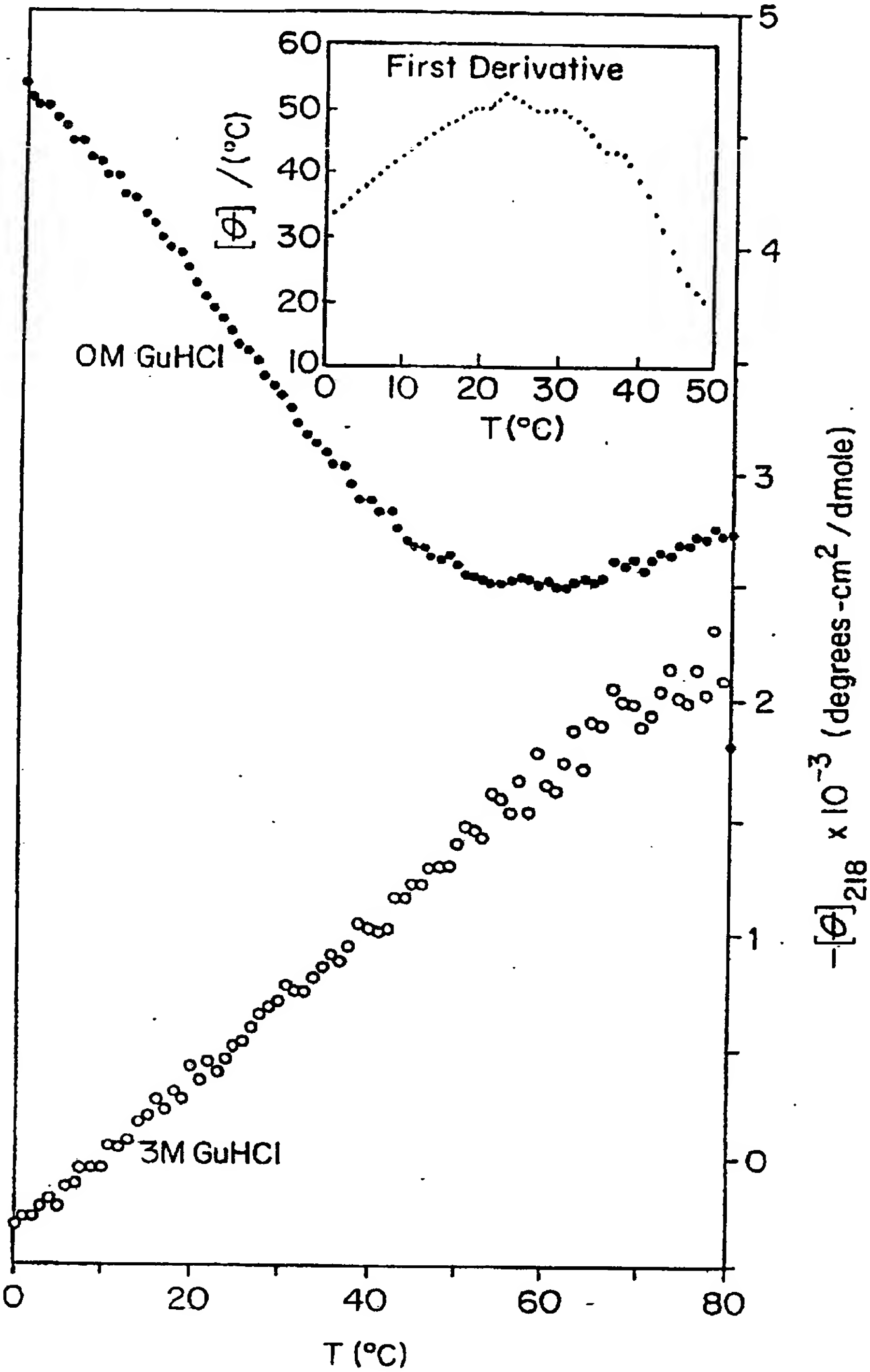

First Derivative
[Φ] /(°C)
60
50
40
30
20
10
0
10
20
30
40
50
T(°C)
OM GuHCl
3M GuHCl
$-[\Phi]_{218} \times 10^{-3}$ (degrees-cm^2/dmole)
5
4
3
2
1
0
0
20
40
60
80
T (°C)

Fig. 8

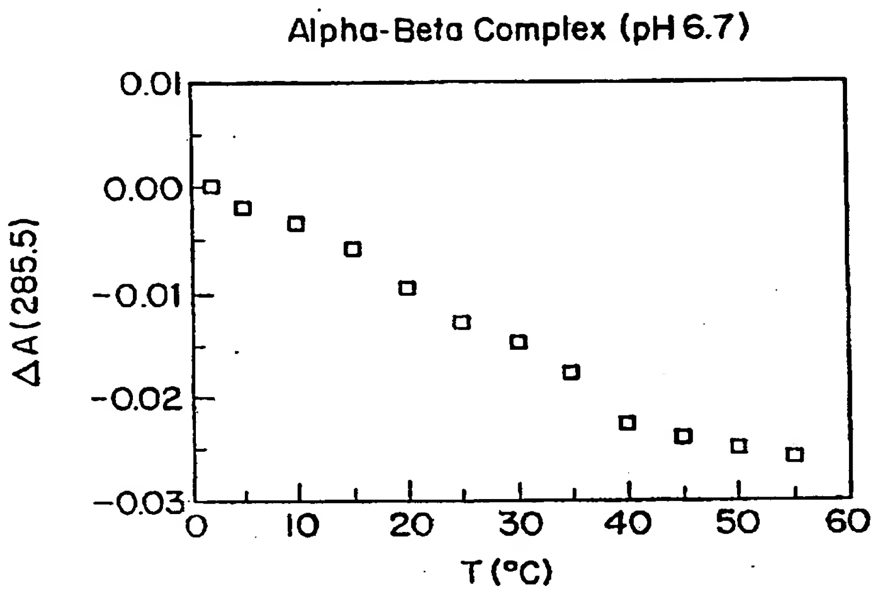

Alpha-Beta Complex (pH 6.7)
ΔA (285.5)
0.01
0.00
-0.01
-0.02
-0.03
0
10
20
30
40
50
60
T (°C)

Fig. 9

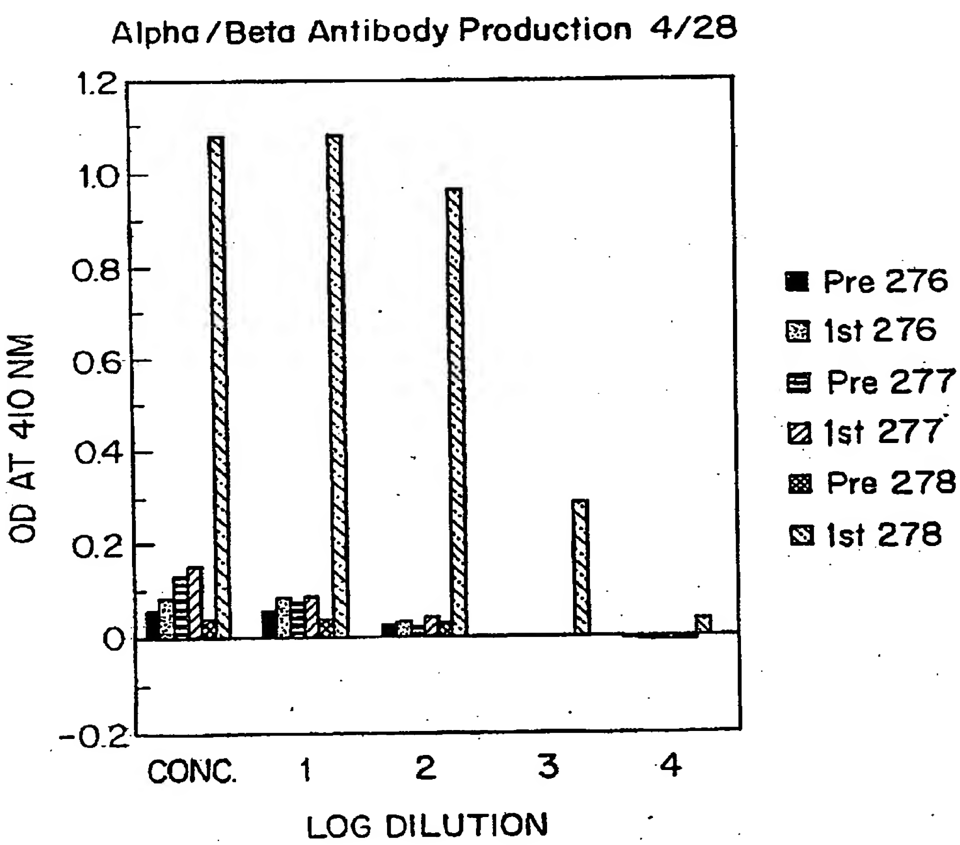

Alpha/Beta Antibody Production 4/28
OD AT 410 NM
1.2
1.0
0.8
0.6
0.4
0.2
0
-0.2
CONC.
1
2
3
4
LOG DILUTION
Pre 276
1st 276
Pre 277
1st 277
Pre 278
1st 278

Fig. 11

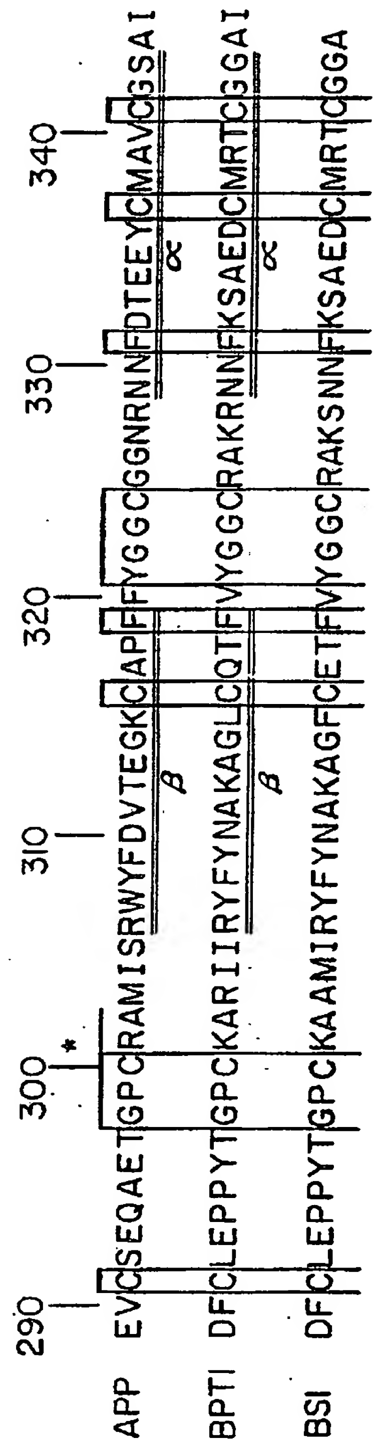

290 300 310 320 330 340
*
APP EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI
β α
BPTI DFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGAI
β α
BSI DFCLEPPYTGPCKAAMIRYFYNAKAGFCETFVYGGCRAKSNNFKSAEDCMRTCGGA

Fig. 10

INTERNATIONAL SEARCH REPORT

International Application No PCT/US88/03033

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 07 K 7/08, C 07 K 7/10, C 07 K 1/00, A 61 K 39/00, G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 85/04103 (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 26 September 1985 see the whole document --	1-6
X	EP, A, 0 229 723 (NOVO INDUSTRI A/S) 22 July 1987 see the whole document --	1-6
P,A	Proc. Natl. Acad. Sci. USA, Vol. 85, March 1988, pages 1662-1666, S. J. Richman et al.: "Immunologic modeling of a 75-kDa malarial protein with carrier-free synthetic peptides", see the whole document --------	1-15

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th December 1988	03 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

PCT/US88/03033
SA 24340

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 02/11/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8504103	26-09-85	EP-A- 0155146	18-09-85
		US-A- 4599231	08-07-86
		JP-T-61501705	14-08-86
		US-A- 4599230	08-07-86
EP-A- 0229723	22-07-87	JP-A-62246596	27-10-87